

**Identification of the hepatic efflux transporters of organic anions using
double transfected MDCKII cells expressing human OATP1B1/MRP2,
OATP1B1/MDR1 and OATP1B1/BCRP**

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Running title: Hepatic efflux transport of anions by MDR1, MRP2 and BCRP

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The number of text pages: 40

The number of figures: 7, and references: 37

The number of words in Abstract: 250 words, Introduction: 675 words, and

Discussion: 1149 words

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance
protein; CER, cerivastatin; CMV, canalicular membrane vesicle; DMEM,
Dulbecco's modified Eagle's medium; E3040,

6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; EBV, Epstein Barr virus; EG, estradiol-17 β -D-glucuronide; EHBR, Eisai hyperbilirubinemic rats; ES, estrone-3-sulfate; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance; MRP, multidrug resistance associated protein; OATP, organic anion transporting polypeptide; PRA, pravastatin; PS, permeability surface product; SD, Sprague-Dawley

Abstract

Until recently, it was generally believed that the transport of various organic anions across the bile canalicular membrane was mainly mediated by multidrug resistance associated protein 2 (MRP2/ABCC2). However, a number of new reports have shown that some organic anions are also substrates of multidrug resistance 1 (MDR1/ABCB1) and/or breast cancer resistance protein (BCRP/ABCG2), implying MDR1 and BCRP could also be involved in the biliary excretion of organic anions in humans. In the present study, we constructed new double transfected Madin-Darby canine kidney II (MDCKII) cells expressing organic anion transporting polypeptide 1B1 (OATP1B1)/MDR1 and OATP1B1/BCRP, and we investigated the transcellular transport of four kinds of organic anions, estradiol-17 β -D-glucuronide (EG), estrone-3-sulfate (ES), pravastatin (PRA) and cerivastatin (CER), to identify which efflux transporters mediate the biliary excretion of compounds using double transfected cells. We observed the vectorial transport of EG and ES in all the double transfectants. MRP2 showed the highest efflux clearance of EG among these efflux transporters, while BCRP-mediated clearance of ES was the highest in these double transfectants. In addition, two kinds of HMG-CoA reductase inhibitors,

CER and PRA, were also substrates of all these efflux transporters. The rank order of the efflux clearance of PRA mediated by each transporter was the same as that of EG, whereas the contribution of MDR1 to the efflux of CER was relatively greater than for PRA. This experimental system is very useful for identifying which transporters are involved in the biliary excretion of organic anions which cannot easily penetrate the plasma membrane.

Introduction

Biliary excretion is one of the major pathways for the elimination of unnecessary compounds from blood circulation. In the common process of hepatic clearance, compounds are taken up into liver, converted to more hydrophilic metabolites by metabolizing enzymes responsible for oxidation (e.g. cytochrome P450) and/or conjugation (e.g. UDP-glucuronosyl transferases and sulfotransferases) and subsequently excreted into bile. Several kinds of ATP-binding cassette (ABC) transporters on the bile canalicular membrane play an important role in this biliary excretion. It is generally accepted that multidrug resistance associated protein 2 (MRP2/ABCC2) is responsible for the biliary excretion of a wide variety of organic anions including glutathione and glucuronide conjugates (Suzuki and Sugiyama, 1998; Konig et al., 1999). This has been proven by comparing the transport activity across the bile canalicular membrane between normal and MRP2-deficient rats such as Eisai hyperbilirubinemic rats (EHBR) or TR⁻ rats (Suzuki and Sugiyama, 1998; Konig et al., 1999).

Recently, it has been found that some organic anions can also be substrates of other ABC transporters. Multidrug resistance 1 (MDR1/ABCB1)

preferentially accepts hydrophobic cationic or neutral compounds (Tanigawara, 2000; Varadi et al., 2002). However, Cvetkovic et al. (1999) reported that fexofenadine, an anionic nonsedating antihistamine, could be transported by human MDR1 (Cvetkovic et al., 1999). It has also been reported that estradiol-17 β -D-glucuronide (EG) is a substrate of MDR1 as well as MRP2 in humans (Huang et al., 1998). Regarding the sulfated conjugates, we previously found that the biliary excretion clearance of the sulfates of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) and liquiritigenin were excreted into bile in EHBR to the same extent as in Sprague-Dawley (SD) rats (Shimamura et al., 1994; Takenaka et al., 1995), suggesting that biliary excretion of sulfate-conjugates is not mainly mediated by MRP2 in rats. Suzuki et al. (2003) demonstrated that breast cancer resistance protein (BCRP/ABCG2) accepts various kinds of organic anions and preferentially transports sulfate conjugates (Suzuki et al., 2003). Taking into consideration the finding that MDR1 and BCRP are expressed in the canalicular membrane (Thiebaut et al., 1987; Maliepaard et al., 2001) in addition to these other facts, not only MRP2 but also MDR1 and BCRP can be involved in the biliary excretion of organic anions.

The double transfected Madin-Darby canine kidney II (MDCKII) cell lines expressing both organic anion transporting polypeptide 1B1 (OATP1B1/OATP-C/OATP2) or OATP1B3 (OATP8) in the basolateral membrane and MRP2 in the apical membrane, have been established as an *in vitro* model of hepatic vectorial transport of organic anions in humans (Cui et al., 2001; Sasaki et al., 2002). In this system, we can observe clear vectorial transport of bisubstrates for uptake and efflux transporters from the basal to the apical side compared with that in the opposite direction. The advantage of this double transfectant system is that it is able to evaluate the transport activities of apical transporters more sensitively compared with membrane vesicles. For example, with pravastatin (PRA), an anionic HMG-CoA reductase inhibitor, the ATP-dependent uptake is very small in human canalicular membrane vesicles (CMVs) (Niinuma et al., 1999), whereas the transcellular transport activity of PRA in OATP1B1/MRP2 double transfectant is large enough to observe its saturation kinetics (Sasaki et al., 2002).

In the present study, we constructed new double transfectants, expressing OATP1B1/MDR1 and OATP1B1/BCRP, and investigated the transcellular transport of organic anions to determine which transporters are involved in the

biliary excretion. OATP1B1 is exclusively expressed in human liver and accepts many kinds of organic anions (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000) and, very recently, Hirano et al. (2004) showed that pitavastatin and EG are mainly taken up by OATP1B1 across the human basolateral membrane (Hirano et al., 2004). Accordingly, ligands can efficiently reach the efflux transporters from the intracellular compartment via OATP1B1, which makes this system useful for the characterization of the efflux transport of organic anions on the bile canalicular membranes. We investigated the transcellular transport of the following organic anions, EG and estrone-3-sulfate (ES), and HMG-CoA reductase inhibitors, cerivastatin (CER) and PRA, to examine whether these compounds exhibited vectorial basal-to-apical transport in each double transfectant or not.

Materials and Methods

Materials. [^3H]-EG (1.6 TBq/mmol) and [^3H]-ES (2.2 TBq/mmol) were purchased from NEN Life Science Products (Boston, MA). [^3H]-CER (0.18 TBq/mmol) was synthesized by Hartmann Analytic GmbH (Braunschweig, Germany). [^3H]-PRA (1.6 TBq/mmol) was kindly donated by Sankyo Co. Ltd. (Tokyo, Japan). Unlabelled CER was kindly donated by Bayer AG (Wuppertal, Germany). pEB6CAGMCS/SRZeo was kindly donated by Dr. Miwa (Tsukuba Univ., Japan) (Tanaka et al., 1999). Parental MDCKII cells and MDCKII cells expressing human MRP2 (Evers et al., 1998) and MDR1 were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, the Netherlands). All other chemicals were commercially available and of reagent grade.

Construction of plasmid vector. Previously cloned human OATP1B1 cDNA in pcDNA3.1/Zeo (+) vector (Iwai et al., 2004) was subcloned into the NotI and XhoI sites of pEB6CAGMCS/SRZeo vector, which is an Epstein barr virus (EBV)-based vector and the subcloned gene was localized and replicated in the episome and not integrated into the genome of the host cells (Tanaka et al., 1999).

Cell culture and transfection of expression vector. Parental MDCKII cells

and MDCKII cells expressing human MRP2 or MDR1 were cultured in Dulbecco's modified Eagle's medium (low glucose version) (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma, St. Louis, MO) and 1% Antibiotic-Antimycotic solution (Sigma) at 37 °C under 5% CO₂. The transporter cDNA in the episomal expression vector was transfected into MDCKII cells using FuGENE6 reagent (Roche Diagnostics Co., Indianapolis, IN). At 50% confluence, cells on 6-well plates were exposed to serum-free DMEM containing plasmid and FuGENE6, according to the manufacturer's instruction. At 6 hr after the initiation of transfection, the plasmid-FuGENE6 solution was replaced with the normal culture medium. The transfected MDCKII cells were selected with Zeocin (700 µg/ml; Invitrogen).

Construction of human BCRP expressing cells. For constructing MDCKII cells expressing human BCRP, MDCKII cells were infected with recombinant adenoviruses containing human BCRP cDNA (Kondo et al., 2004) at 200 moi, 48 hr prior to all experiments. The virus titer was determined as described previously (Kondo et al., 2004).

Western blot analysis. For Western blot analysis, crude membrane was prepared from MDCKII cells according to the method of the previous report

(Gant et al., 1991). After the crude membrane was suspended in PBS, it was frozen in liquid N₂ and stored at -80 °C until used. The protein concentrations in the crude membrane vesicles prepared from MDCKII cells were determined by the method of Lowry with bovine serum albumin as a standard. The membrane fraction was dissolved in 3 x SDS sample buffer (New England BioLabs, Beverly, MA) with beta-mercaptoethanol and loaded onto a 7 % SDS-polyacrylamide electrophoresis gel with a 4.4 % stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs, Beverly, MA). Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad Laboratories, Hercules, CA) at 15 V for 1 hr. The membrane was blocked with Tris-buffered saline with 0.05 % Tween 20 (TTBS) and 5 % skimmed milk overnight at 4 °C. After washing with TTBS, the membrane was incubated at room temperature in TTBS with 1000-fold diluted anti-OATP1B1 polyclonal antibody (Alpha Diagnostic International Inc., San Antonio, TX) for 1 hr, 125-fold diluted monoclonal antibody against MRP2 (M₂III-6) for 2 hr, 100-fold diluted monoclonal antibody against MDR1 (C219) for 1 hr, or 200-fold diluted monoclonal antibody against BCRP (BXP-21) for 2 hr.

For the detection of each transporter, the membrane was placed in contact with 2500-fold diluted donkey anti-rabbit (OATP1B1) or anti-mouse IgG (MRP2, MDR1, and BCRP) conjugated with the horseradish peroxidase (Amersham Biosciences Inc., Dedham, MA) for 1 hr in TTBS. The band was detected using a ECL Plus Western blotting Starter Kit (Amersham Biosciences Inc., Dedham, MA) and its intensity was quantified in a luminescent image analyzer, (LAS-3000 mini; Fuji Film Corp., Tokyo, Japan).

Immunocytochemical staining. For immunocytochemical staining, transfectants were plated at a density of 5×10^5 cells in 12-well plates, 96 hr prior to the experiments. Sodium butyrate (5 mM) was added to the culture medium one day before the experiments. After fixation with methanol at -20°C for 10 min and permeabilization in 1% TritonX-100 in PBS at room temperature for 10 min, cells were incubated for 1 hr at room temperature with 50-fold diluted anti-OATP1B1 rabbit serum, which was raised in rabbits against the 21 amino acids at the carboxyl terminus of the deduced OATP1B1 sequence (ESLNKNKHFVPSAGADSETHC), 40-fold diluted monoclonal antibody against MRP2 (M₂III-6, Alexis Biochemicals, Gruenberg, Germany), 40-fold diluted the monoclonal antibody against MDR1 (C219, SIGNET Laboratories Inc., Dedham,

MA), or 40-fold diluted monoclonal antibody against BCRP (BXP-21, Kamiya Biomedical Company, Seattle, WA). Then, cells were washed with PBS three times and incubated for 1 hr at room temperature with 250-fold diluted Goat anti-rabbit IgG Alexa 488 (Molecular Probes Inc., Eugene, OR) for OATP1B1 or 250-fold diluted Goat anti-mouse IgG Alexa 568 (Molecular Probes Inc., Eugene, OR) for MRP2, MDR1 and BCRP. Nuclei were stained with 250-fold diluted TO-PRO-3 iodide (Molecular Probes Inc., Eugene, OR). The localization was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).

Transcellular transport study. The transcellular transport study was performed as reported previously (Sasaki et al., 2002). Briefly, MDCKII cells were grown on Transwell membrane inserts (6.5 mm diameter, 0.4 μ m pore size; Corning Coster, Bodenheim, Germany) at confluence for 3 days and the expression level of transporters was induced with 5 mM sodium butyrate for 24 hr before the transport study. Cells were firstly washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl₂ adjusted to pH 7.4) at 37 °C or 4 °C. Subsequently, radiolabeled substrates were added in

Krebs-Henseleit buffer either to the apical compartments (250 μ l) or to the basolateral compartments (1 ml). After a designated period, the radioactivity in 100 μ l medium in the opposite compartments was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Inc., Fullerton, CA). At the end of the experiments, the cells were washed three times with 1.5 ml ice-cold Krebs-Henseleit buffer and solubilized in 500 μ l 0.2 M NaOH. After addition of 100 μ l 1 M HCl, 450 μ l aliquots were transferred to the liquid scintillation counter for determination of radioactivity. 50 μ l aliquots of cell lysate were used to determine protein concentrations by the method of Lowry with BSA as a standard.

Calculation of the transport activities of recombinant MRP2, MDR1, and BCRP across the double transfectants. The apparent efflux clearance across the apical membrane ($PS_{apical, x^{\circ}C}$) at each temperature (37°C or 4°C) was calculated by dividing the steady-state velocity for the transcellular transport ($V_{transcellular, x^{\circ}C}$) of compounds determined over 2 hr by the cellular concentration ($C_{cell, x^{\circ}C}$) of the compounds determined at the end of the experiments (over 2 hr) at each temperature (37°C or 4°C). The steady-state velocity for the transcellular transport was calculated by dividing the basal-to-apical transport

amount at 2 hr ($X_{x^{\circ}C}$) when the steady-state condition was maintained (see **Results**) by 120 (minutes). Then, to evaluate the activity of transporter-mediated transcellular transport, $PS_{apical, 4^{\circ}C}$, the clearance at 4°C (at this temperature the active transport systems are not functional.) was subtracted from $PS_{apical, 37^{\circ}C}$. Moreover, since some endogenous efflux transporters have been reported to be expressed in MDCKII cells (Goh et al., 2002; Guo et al., 2002), the specific transport activity across the apical membrane (TA) mediated by exogenously-expressed efflux transporters was calculated by subtracting the transporter-mediated clearance in each double transfectant from that in the single transfectant expressing only OATP1B1, as described in the following equations:

$$PS_{apical, x^{\circ}C} = \frac{V_{transcellular, x^{\circ}C}}{C_{cell, x^{\circ}C}} \quad (\text{eq. 1})$$

$$TA = (PS_{apical, 37^{\circ}C, double} - PS_{apical, 4^{\circ}C, double}) - (PS_{apical, 37^{\circ}C, single} - PS_{apical, 4^{\circ}C, single}) \quad (\text{eq. 2})$$

Results

Expression of human OATP1B1, MRP2, MDR1 and BCRP in MDCKII cells.

The expression of OATP1B1, MRP2, MDR1 and BCRP in double transfectants was confirmed by Western blot analysis (Figure.1). Two major bands, which appeared at 83 kDa and 62 kDa, could be detected in all kinds of OATP1B1-transfected cells (Figure.1 A), as shown previously (Konig et al., 2000). We were able to clearly detect human MRP2, MDR1 and BCRP with apparent molecular masses of about 190 kDa, 170 kDa and 70 kDa (Figure.1 B~D). The band also reacted slightly with the C219 antibody in wild-type MDCKII cells (Figure.1 C).

Localization of recombinant human OATP1B1, MRP2, MDR1 and BCRP.

The cellular localization of the recombinant transporters in each transfectant was confirmed by confocal laser scanning microscopy. OATP1B1 was localized in the basolateral membrane (Figure.2 B, F~H), while MRP2 and MDR1 were localized in the apical membrane (Figure.2 C, D, F and G). BCRP was mainly detected in the apical membrane but part of the BCRP was also detected in the basolateral membrane (Figure.2 E, H).

Transcellular transport of EG, ES, CER and PRA across the MDCKII cell

monolayer.

Transcellular transport of conjugated steroids, EG and ES across the MDCKII monolayer was determined. As shown in Figure.3 F~H, the basal-to-apical transport of EG was approximately 17, 6.7 and 8.8 times higher than that in the opposite direction in OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP double transfectants, respectively, while the basal to apical flux of EG across the OATP1B1-expressing MDCKII cells was approximately 2.3 times higher than that in the opposite direction (Figure.3 B). A symmetrical flux of EG was observed across the control and MRP2, MDR1 and BCRP-expressing MDCKII cells (Figure.3 A, C~E). For ES, the basal-to-apical transport was approximately 2.2, 3.0, 10 and 41-fold higher than that in the opposite direction in OATP1B1, OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP-expressing MDCKII cells, respectively (Figure.4 B, F~H). On the other hand, a symmetrical flux of ES was observed across the control and MRP2, MDR1 and BCRP-expressing MDCKII cells (Figure.4 A, C~E). Transcellular transport of two kinds of HMG-CoA reductase inhibitors, CER and PRA, was also determined in the MDCKII transfectants. As shown in Figure.5 F~H, the basal-to-apical transport of CER was 3.8, 6.3 and 3.1 times higher than

that in the opposite direction in OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP-expressing MDCKII cells, respectively, while a symmetrical flux of CER was observed across the control and all of the single transfectants (Figure.5 A~E). On the other hand, the basal-to-apical flux of PRA was significantly 3.3-fold higher than that in the opposite direction only in OATP1B1/MRP2-expressing MDCKII cells (Figure.6 F). However, in the other cell lines, the ratio of the basal-to-apical flux to that in the opposite direction was less than two (Figure.6 A~E, G, H).

Calculation of the transport activities of recombinant MRP2, MDR1, and BCRP across the apical membrane of the double transfectants.

In order to estimate quantitatively the transport activity by the recombinant transporters across the apical membrane, the clearance to the apical compartment from cells (*TA*) was determined as described in **Materials and Methods**. The clearance for EG was 3.56 ± 0.07 , 0.420 ± 0.026 , 0.383 ± 0.059 in OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP-expressing MDCKII cells, respectively (Figure.7 A). The clearance for ES was 0.268 ± 0.013 , 0.351 ± 0.011 , 2.31 ± 0.02 in OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP-expressing MDCKII cells, respectively (Figure.7 B).

Regarding the statins, the clearance for CER was 0.612 ± 0.039 , 0.669 ± 0.062 , 0.201 ± 0.007 in OATP1B1/MRP2, OATP1B1/MDR1 and OATP1B1/BCRP-expressing MDCKII cells, respectively (Figure.7 C), whereas the clearance for PRA was 3.75 ± 0.112 , 0.393 ± 0.097 , 0.194 ± 0.087 , respectively (Figure.7 D).

Discussion

In the present study, we constructed new double transfectants, expressing OATP1B1/MDR1 and OATP1B1/BCRP and observed the transcellular transport of four organic anions, EG and ES (steroid conjugates), and CER and PRA (HMG-CoA reductase inhibitors) to examine the substrate specificities and relative transport activities of the efflux transporters, MDR1, MRP2 and BCRP.

Western blot and immunocytochemical analyses revealed that OATP1B1 and MRP2 and MDR1 were expressed in MDCKII cells and localized correctly on the basolateral (OATP1B1) and apical membranes (MRP2 and MDR1), respectively, but BCRP was localized mainly on the apical membrane, and only partially on the basolateral membrane (Figure. 2). On the other hand, we could clearly observe significant basal-to-apical vectorial transport of organic anions in OATP1B1/BCRP double transfected cells (Figure.3~5 H), suggesting that minor expression of BCRP on the basal side does not affect the observation of the vectorial transport of substrates with our double transfectant, although basal-to-apical transport was thought to be partly inhibited by the basal expression of BCRP.

Then, we performed a transcellular transport study involving four kinds of

organic anions using three types of double transfectants (Figure.3~6). The double transfectant is a useful tool for identifying bisubstrates for uptake and efflux transporters and suitable for high throughput screening (Sasaki et al., 2002). Another advantage of this system is to characterize the function of efflux transporters easily because some compounds cannot access the efflux transporter from the intracellular compartment without the aid of uptake transporters. In this study, all test compounds are known to be substrates of OATP1B1 (Abe et al., 1999; Hsiang et al., 1999; Shitara et al., 2003) and compounds can interact with efflux transporters efficiently.

The transcellular transport clearance (PS_{trans}) was determined by both uptake and efflux clearance as shown in eq. (3).

$$PS_{trans} = PS_{uptake} \times \frac{PS_{apical}}{PS_{apical} + PS_{basal}} \quad \text{eq. (3)}$$

, where PS_{uptake} represents the uptake clearance from the basal side to the cell and PS_{apical} and PS_{basal} represent the efflux clearance from the cell to the apical side and to the basal side, respectively. If PS_{apical} is much larger than PS_{basal} , which is a typical case when the efflux transporter can recognize the substrate, PS_{trans} is approximately equal to PS_{uptake} , which is determined by the function of OATP1B1. So, the transcellular transport clearance does not always reflect the

function of the efflux transporter. Therefore, to estimate the relative clearance of each efflux transporter, we calculated the PS_{apical} values by transcellular clearance from the basal to apical side normalized by the intracellular ligand concentration (Figure.7).

EG was efficiently transported from the basolateral to apical side in all these double transfectants (Figure.3) and the efflux clearance of MRP2 was much higher than that of MDR1 and BCRP (Figure.7 A). We previously reported that Mrp2 is predominantly involved in the biliary excretion of EG in rats (Morikawa et al., 2000). It has also been reported that EG is a substrate of human MDR1 and BCRP (Huang et al., 1998; Chen et al., 2003).

We were also able to observe the vectorial transcellular transport of ES in all kinds of double transfectants and efflux transporters were able to enhance the basal-to-apical transport of ES compared with the transport in OATP1B1-single transfectant. BCRP showed the highest efflux clearance of ES among the three transporters (Figure.7 B). Suzuki et al. (2003) reported that BCRP preferentially transports sulfated conjugates (Suzuki et al., 2003) and our results suggest that the sulfate-conjugated steroid, ES, could be transported preferentially by BCRP in our double transfectants compared with MDR1 and

MRP2. This result agrees with the previous report demonstrating that the biliary excretion of the sulfated steroid, E3040-sulfate, was maintained even in EHBR, an Mrp2-deficient rat (Takenaka et al., 1995)

HMG-CoA reductase inhibitors (statins) are efficiently taken up into the liver, where cholesterol is synthesized (Igel et al., 2001). Among these statins, PRA, CER, pitavastatin and rosuvastatin are reported to be substrates of OATP1B1 (Brown et al., 2001; Nakai et al., 2001; Sasaki et al., 2002; Shitara et al., 2003; Hirano et al., 2004). The basal-to-apical flux of CER was significantly higher than that in the opposite direction in all the double transfectants compared with the OATP1B1-single transfectant (Figure.5), suggesting that CER is a substrate of MRP2, MDR1 and BCRP. A previous report has demonstrated that the transcellular transport of CER from the basal to apical side across the MDR1-expressed MDCK monolayer was significantly greater than that in the opposite direction (Hirai et al., 2001). We were also able to obtain reproducible results in the MDR1-single transfectant (Figure.5 G). On the other hand, the ratio of the basal-to-apical flux to the apical-to-basal flux of PRA was significantly high only in the OATP1B1/MRP2 double transfectant, whereas the flux ratio was only slightly raised in OATP1B1/MDR1 and OATP1B1/BCRP transfectants

(Figure.6). Previous reports have suggested that the biliary excretion of PRA was mostly mediated by rat Mrp2 (Yamazaki et al., 1997). Interestingly, the relative activities of each transporter for PRA were very similar to those for EG and previous reports have demonstrated that Mrp2 is responsible for the biliary excretion of both PRA and EG in rats, suggesting that PRA and EG may share the same route of biliary excretion, possibly Mrp2. However, considering the reported species differences in the expression level of transporters (Ishizuka et al., 1999), we cannot easily estimate the relative contribution of each efflux transporter from our own data alone and will need further analyses in which we compare the expression level of apically-localized transporters in double transfectants and human liver accurately. In our expression system, we observed a low degree of MDR1-mediated transport of PRA. Some reports have shown that PRA does not interact with MDR1 using a cell system (Wang et al., 2001; Sakaeda et al., 2002; Hirai et al., 2001). However, it is possible that the intracellular concentration of PRA was too low for them to detect MDR1-mediated efflux of PRA because PRA cannot cross the basal membrane in the MDR1-single transfectant.

In conclusion, we have constructed new double transfected cell lines and

determined the substrate specificities and relative transport activities of MRP2, MDR1, and BCRP for organic anions. Vectorial transport of EG, ES, CER and PRA from the basal to apical side was observed in OATP1B1/MRP2-, OATP1B1/MDR1-, and OATP1B1/BCRP-expressing cells. The transport activities of EG and PRA by MRP2 was the highest, considering that MRP2 may be mainly involved in the transport of EG and PRA in humans as well as rodents. In the case of ES, BCRP may play an important role in the biliary excretion. It is interesting that two kinds of structurally-related HMG-CoA reductase inhibitors, PRA and CER, showed different relative contributions from each transporter as far as biliary excretion is concerned. This system is useful for the determination of the substrate specificities of hepatic efflux transporters for hydrophilic organic anions, which cannot easily be extruded from the cellular membrane. We can also determine the substrate-dependence of the relative contribution of each transporter using this double transfected cell system.

Acknowledgments

We would like to thank Dr. Piet Borst (The Netherlands Cancer Institutes) for providing the MDCKII cells expressing MRP2 or MDR1, Sankyo Co. (Tokyo, Japan) for providing labeled and unlabeled pravastatin, Bayer HealthCare AG (Wuppertal, Germany) for unlabelled CER, and Dr. Yoshihiro Miwa (Univ. Tsukuba, Japan) for pEB6CAGMCS/SRZeo vector.

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Footnotes

This study was supported by Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare for the Research on Advanced Medical Technology and by Grant-in Aid for Young Scientists B (15790087) from the Ministry of Education, Culture, Sports, Science, and Technology.

Legends for Figures

Figure 1. Western blot analysis of OATP1B1, MRP2, MDR1 and BCRP in crude membrane vesicles obtained from MDCKII transfectants. Crude membrane prepared from MDCKII transfectants was separated by SDS-PAGE. OATP1B1 was detected using polyclonal antibody against the carboxyl terminus of human OATP1B1 (A). MRP2, MDR1 and BCRP were detected using monoclonal antibody against the linker region of human MRP2 (B), MDR1 (C) and BCRP (D), respectively. The amount of protein applied to each lane in panel (A), (B), (C) and (D) was 100, 2, 20 and 0.25 μ g. C, S1~4, W1~3 represent vector-transfected MDCKII cells, single transfected cells (S1: OATP1B1, S2: MRP2, S3: MDR1, S4: BCRP) and double transfected cells (W1: OATP1B1/MRP2, W2: OATP1B1/MDR1, W3: OATP1B1/BCRP).

Figure 2. Immunolocalization of recombinant OATP1B1, MRP2, MDR1 and BCRP in MDCKII cells. MDCKII cells transfected with empty vector (A), OATP1B1 (B), MRP2 (C), MDR1 (D), BCRP (E), both OATP1B1 and MRP2 (F), both OATP1B1 and MDR1 (G), and both OATP1B1 and BCRP (H) were stained with polyclonal antiserum against human OATP1B1 (*green fluorescence*, A~H),

monoclonal antibody against human MRP2 (*red fluorescence*, A~C, and F), human MDR1 (*red fluorescence*, A, B, D, and G) and human BCRP (*red fluorescence*, A, B, E, and H). Figure 3 (A), (B) shows the staining with antiserum against human OATP1B1 and MDR1. The results were similar to the staining with antiserum against human OATP1B1/MRP2 or OATP1B1/BCRP (data not shown). Nuclei were stained with TO-PRO-3 (*blue fluorescence*). Pictures are single optical sections (*x,y*) (center) with *xz* - (top) and *yz* - (right) projections, respectively. Bar = 20 μ m.

Figure 3. Time-profiles for the transcellular transport of [³H]-EG across MDCKII monolayers. Transcellular transport of [³H]-EG (0.5 μ M) across MDCKII monolayers expressing OATP1B1 (B), MRP2 (C), MDR1 (D), BCRP (E), both OATP1B1 and MRP2 (F), both OATP1B1 and MDR1 (G) and both OATP1B1 and BCRP (H) was compared with that across the control MDCKII monolayer (A). *Open and closed circles* represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each *point* and *vertical bar* represents the mean \pm S.E. of three determinations. Where *vertical bars* are not shown, the S.E. was contained within the limits of the *symbol*.

Figure 4. Time-profiles for the transcellular transport of [³H]-ES across MDCKII monolayers. Transcellular transport of [³H]-ES (0.5μM) across MDCKII monolayers expressing OATP1B1 (B), MRP2 (C), MDR1 (D), BCRP (E), both OATP1B1 and MRP2 (F), both OATP1B1 and MDR1 (G) and both OATP1B1 and BCRP (H) was compared with that across the control MDCKII monolayer (A). *Open and closed circles* represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each *point* and *vertical bar* represent the mean ± S.E. of three determinations. Where *vertical bars* are not shown, the S.E. was contained within the limits of the *symbol*.

Figure 5. Time-profiles for the transcellular transport of [³H]-CER across MDCKII monolayers. Transcellular transport of [³H]-CER (0.5μM) across MDCKII monolayers expressing OATP1B1 (B), MRP2 (C), MDR1 (D), BCRP (E), both OATP1B1 and MRP2 (F), both OATP1B1 and MDR1 (G) and both OATP1B1 and BCRP (H) was compared with that across the control MDCKII monolayer (A). *Open and closed circles* represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each *point* and *vertical bar* represent the mean ± S.E. of three determinations. Where *vertical bars*

bars are not shown, the S.E. was contained within the limits of the *symbol*.

Figure 6. Time-profiles for the transcellular transport of [³H]-PRA across MDCKII monolayers. Transcellular transport of [³H]-PRA (0.5 μ M) across MDCKII monolayers expressing OATP1B1 (B), MRP2 (C), MDR1 (D), BCRP (E), both OATP1B1 and MRP2 (F), both OATP1B1 and MDR1 (G) and both OATP1B1 and BCRP (H) was compared with that across the control MDCKII monolayer (A). *Open* and *closed circles* represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each *point* and *vertical bar* represent the mean \pm S.E. of three determinations. Where *vertical bars* are not shown, the S.E. was contained within the limits of the *symbol*.

Figure 7. Estimation of transporter-mediated efflux activity (TA) for the MRP2, MDR1, and BCRP transfectants. The TA values for EG (A), ES (B), CER (C) and PRA (D) were determined as described in **Materials and Methods**. Values are given as the mean \pm S.E. of three determinations.

Figure. 1

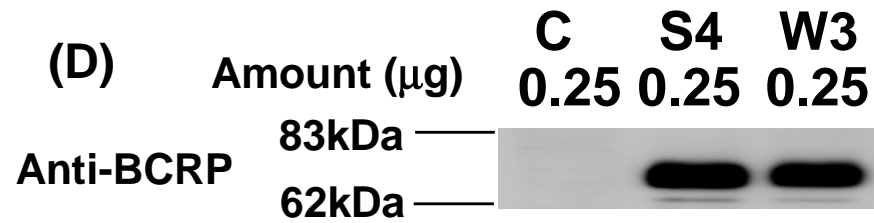
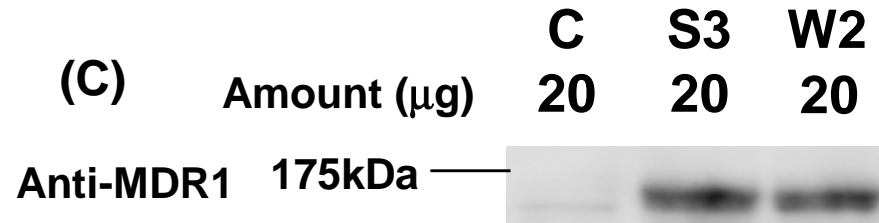
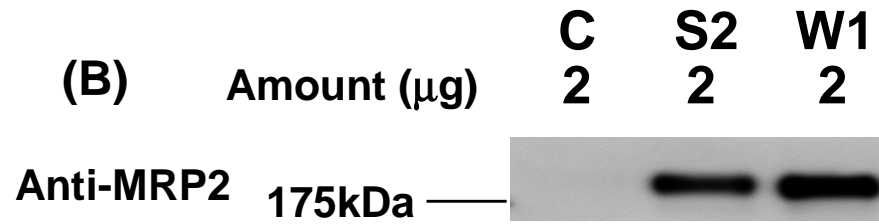
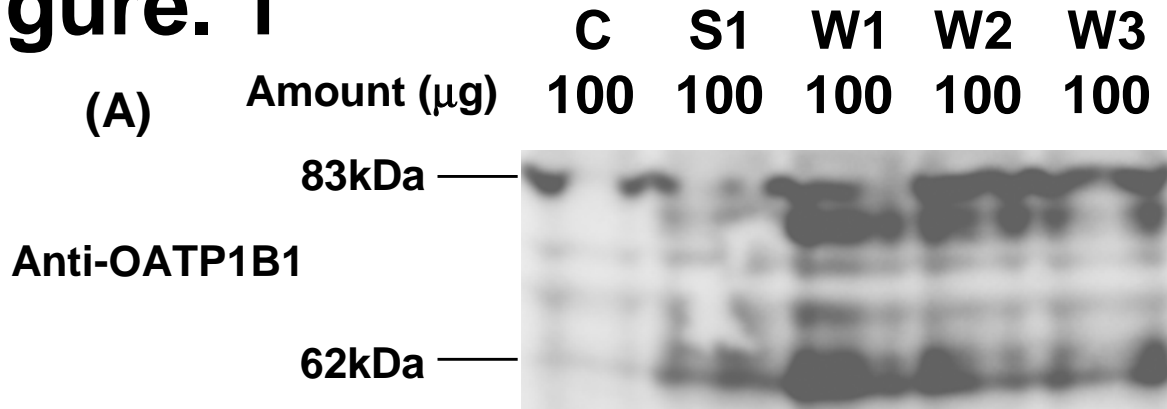


Figure. 2

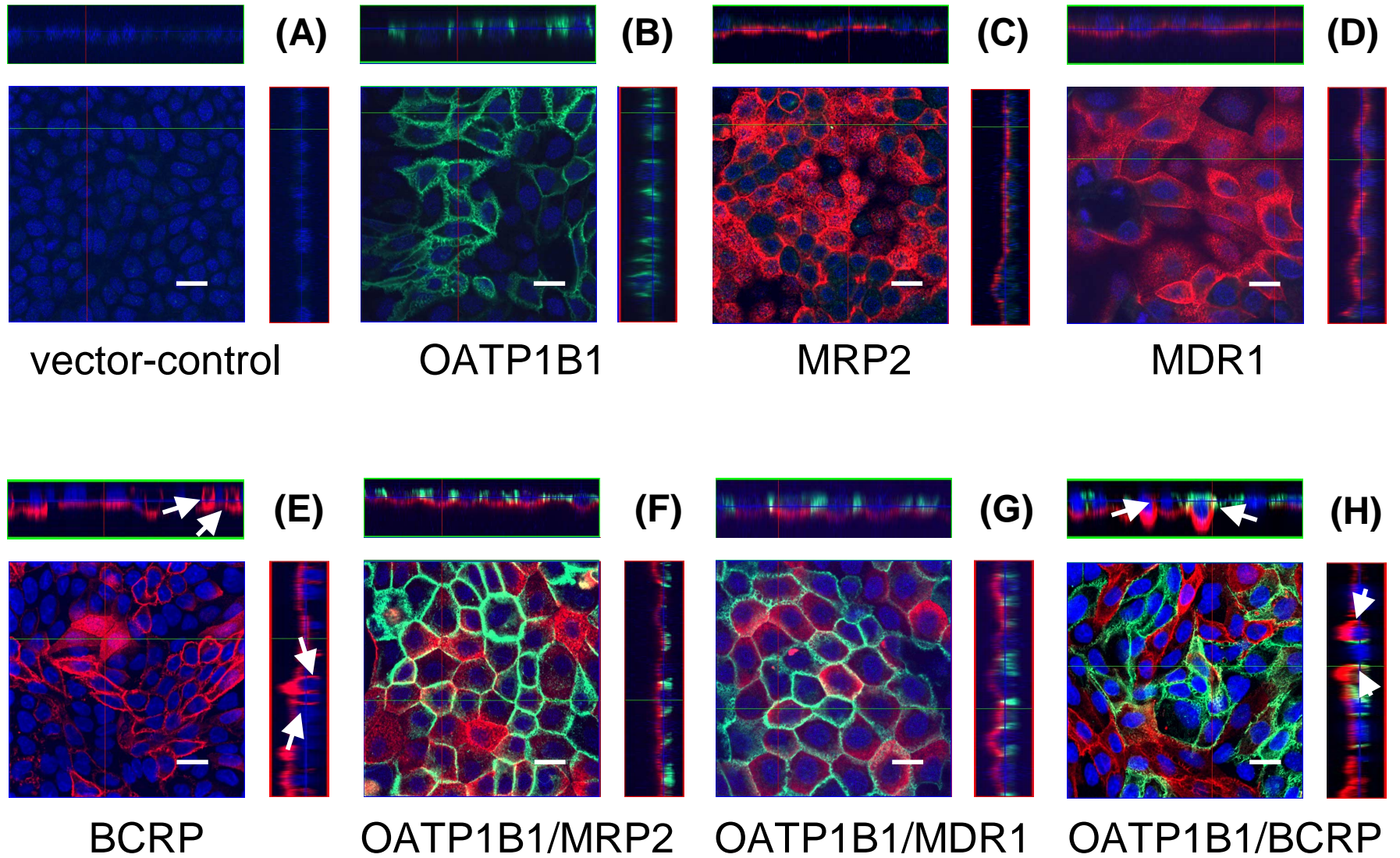


Figure. 3

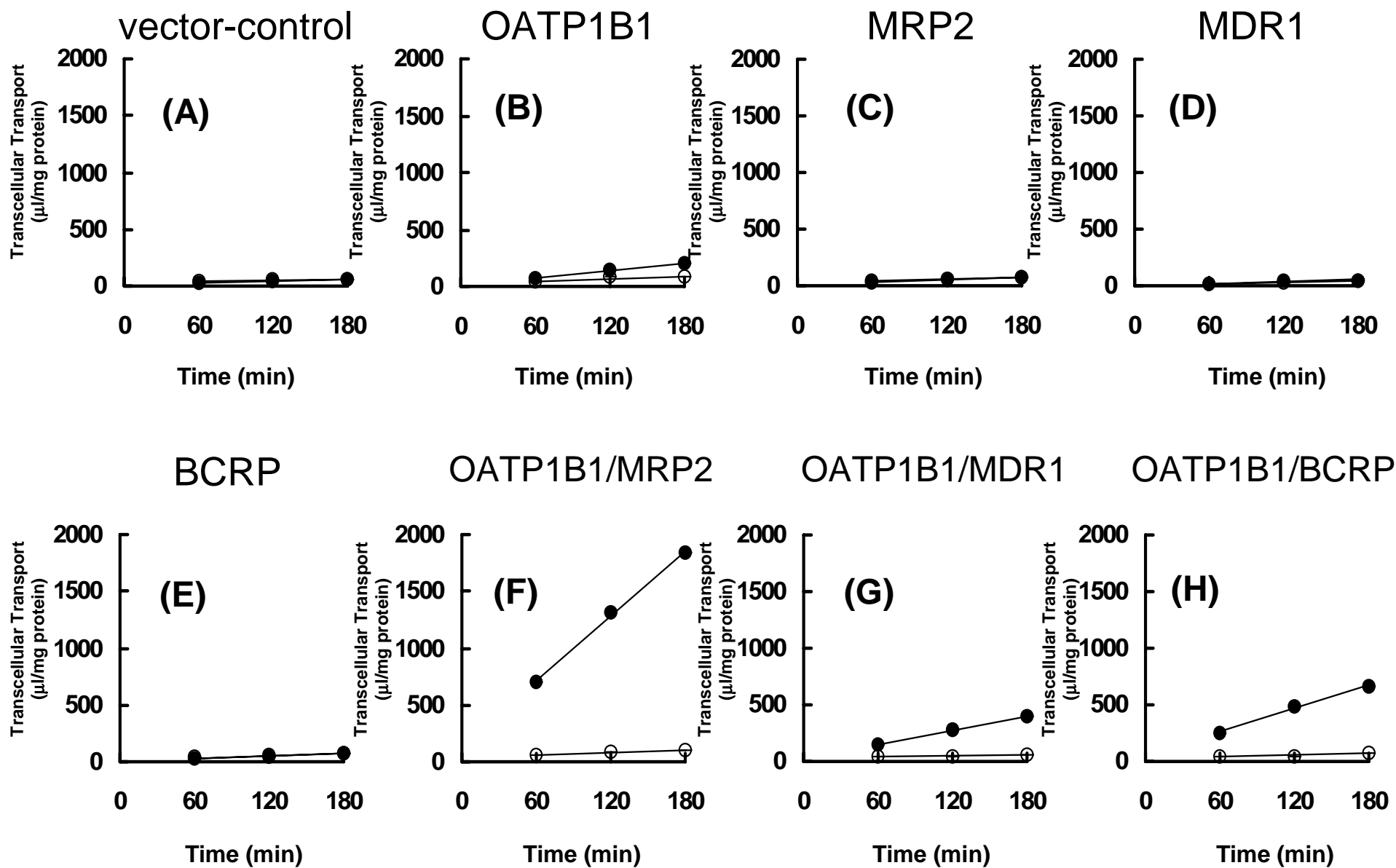


Figure. 4

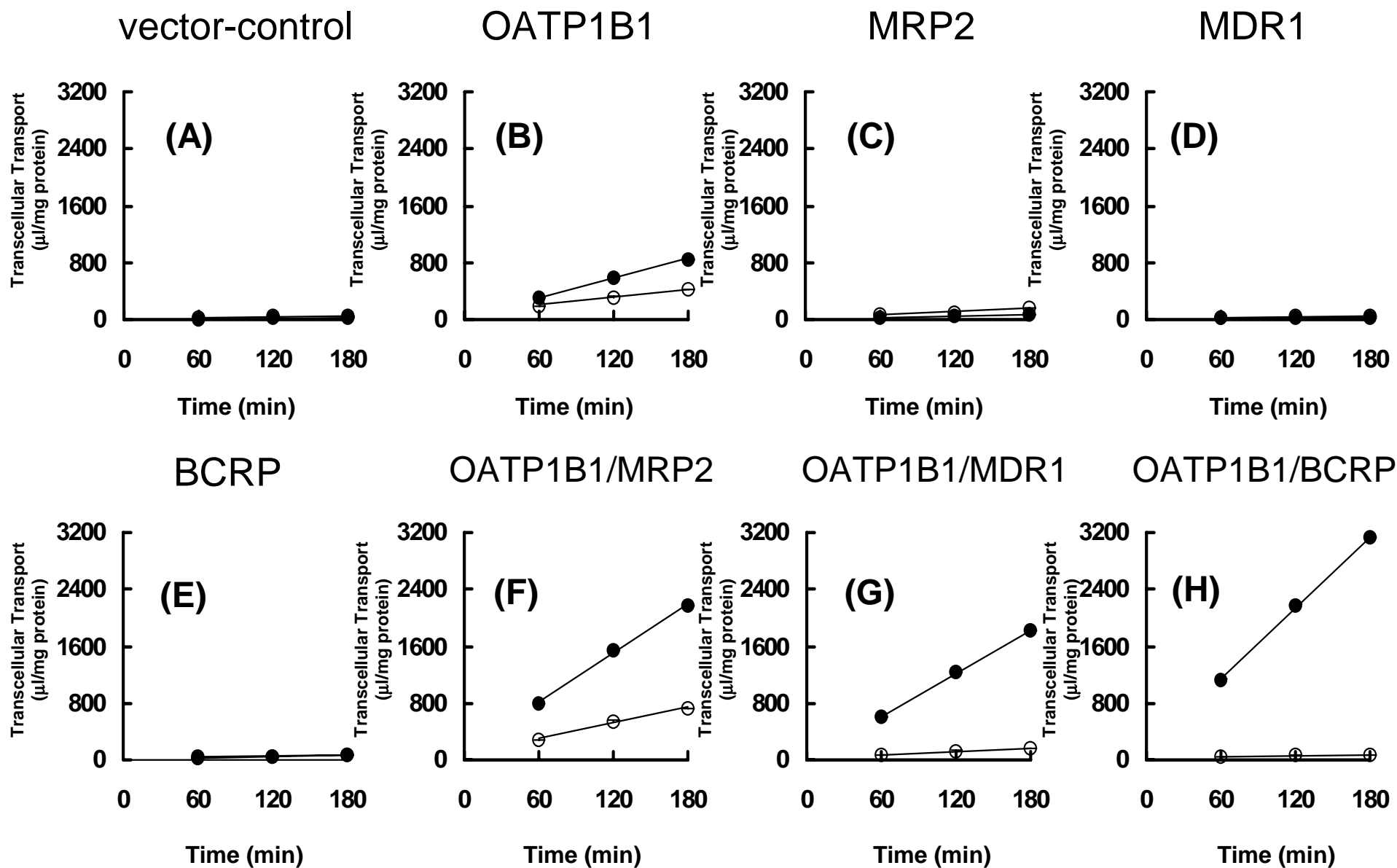


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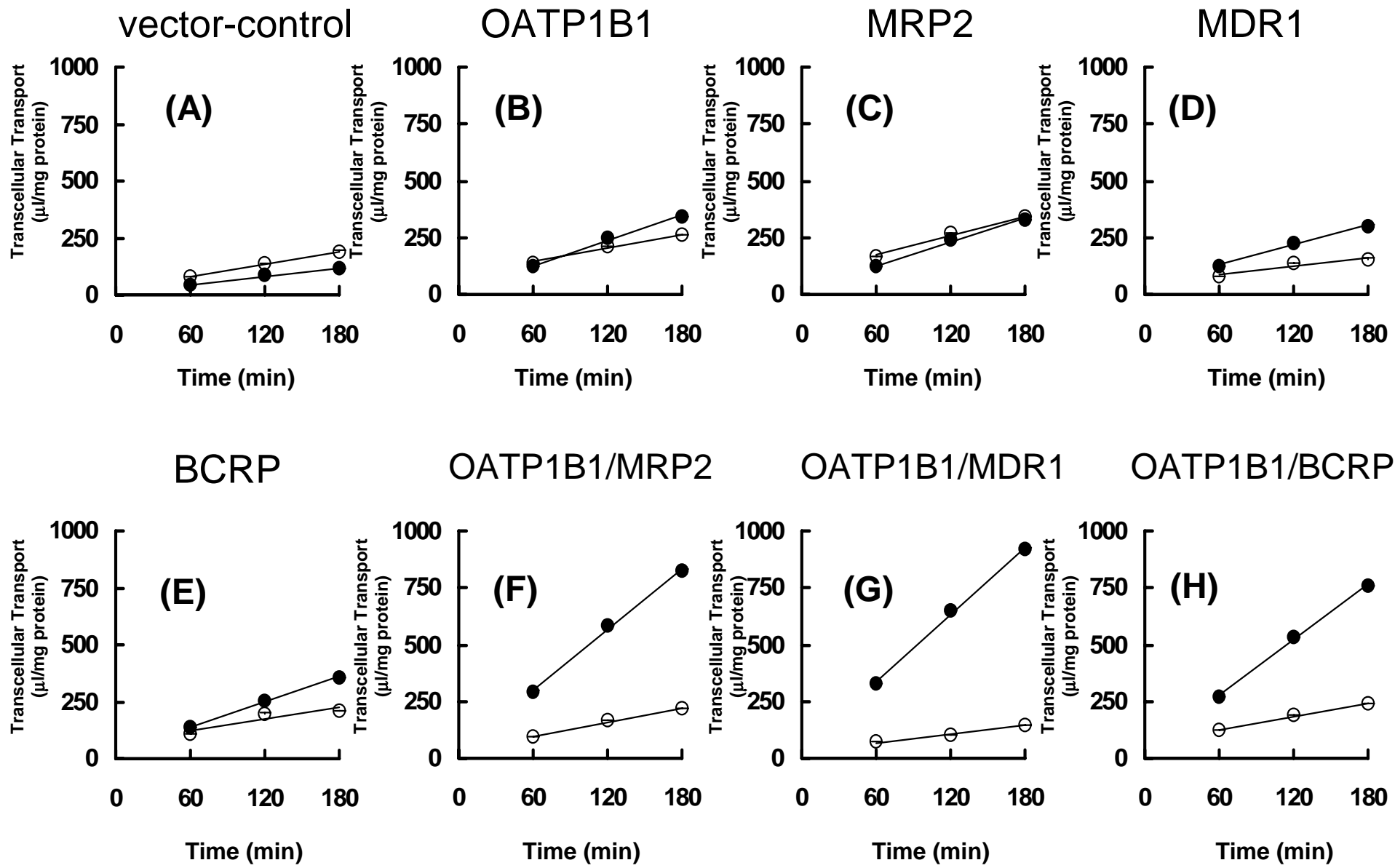


Figure. 6

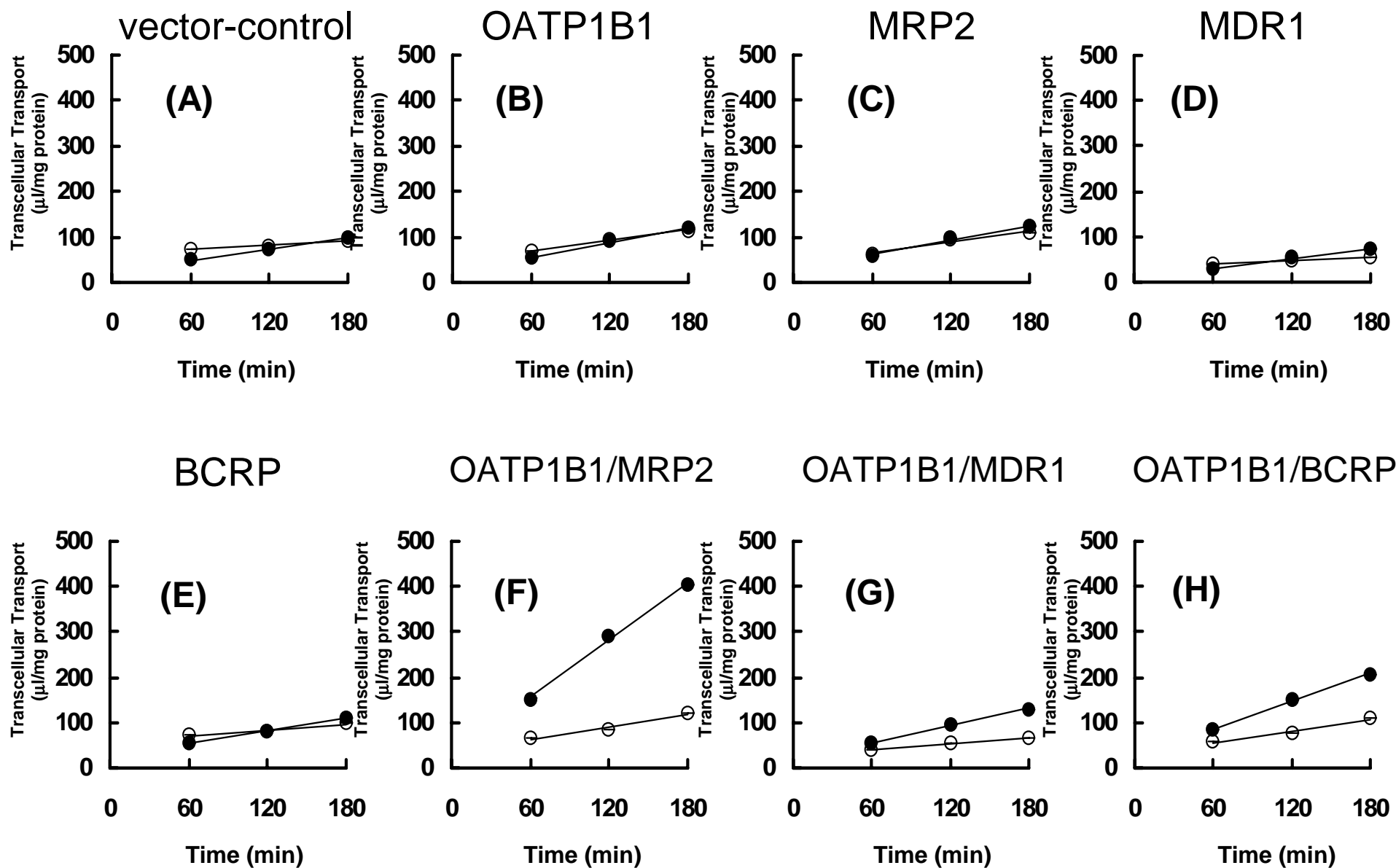


Figure. 7

